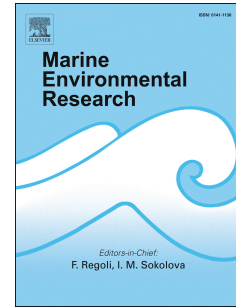


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Interaction strength between different grazers and macroalgae mediated by ocean acidification over warming gradients

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1 **Interaction strength between different grazers and macroalgae mediated by ocean**  
2 **acidification over warming gradients.**

3

4 **Macroalgae-herbivore interactions in changing oceans**

5

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19

20 **Abstract**

21 Since the past century, rising CO<sub>2</sub> levels have led to global changes (ocean warming  
22 and acidification) with subsequent effects on marine ecosystems and organisms.  
23 Macroalgae-herbivore interactions have a main role in the regulation of marine  
24 community structure (top-down control). Gradients of warming prompt complex non-  
25 linear effects on organism metabolism, cascading into altered trophic interactions and  
26 community dynamics. However, not much is known on how will acidification and  
27 grazer assemblage composition shape these effects. Within this context, we aimed to  
28 assess the combined effects of warming gradients and acidification on macroalgae-  
29 herbivore interactions, using three cosmopolitan species, abundant in the Iberian  
30 Peninsula and closely associated in nature: the amphipod *Melita palmata*, the gastropod  
31 *Gibbula umbilicalis*, and the green macroalga *Ulva rigida*. Under two CO<sub>2</sub> treatments  
32 ( $\Delta\text{CO}_2 \approx 450 \mu\text{atm}$ ) across a temperature gradient (13.5, 16.6, 19.9 and 22.1 °C), two  
33 mesocosm experiments were performed to assess grazer consumption rates and  
34 macroalgae-herbivore interaction, respectively. Warming (Experiment I and II) and  
35 acidification (Experiment II) prompted negative effects in grazer's survival and species-  
36 specific differences in consumption rates. *M. palmata* was shown to be the stronger  
37 grazer per biomass (but not per capita), and also the most affected by climate stressors.  
38 Macroalgae-herbivore interaction strength was markedly shaped by the temperature  
39 gradient, while simultaneous acidification lowered thermal optimal threshold. In the  
40 near future, warming and acidification are likely to strengthen top-down control, but  
41 further increases in disturbances may lead to bottom-up regulated communities. Finally,  
42 our results suggest that grazer assemblage composition may modulate future  
43 macroalgae-herbivore interactions.

44

45 Key-words: warming, acidification, macroalgae-herbivore interaction, stressor gradient,  
46 interaction strength.

ACCEPTED MANUSCRIPT

## 47 **Introduction**

48 Anthropogenic CO<sub>2</sub> accumulates in the atmosphere, retaining infrared radiation and  
49 rising temperatures of both terrestrial and ocean ecosystems. As a result, global average  
50 sea surface temperature increased approximately 0.1 °C per decade during the last 40  
51 years (Taboada and Anadón, 2012) and a further 2-5 °C increase in mean sea surface  
52 temperature is predicted by 2100 (IPCC, 2013). Biologically, temperature affects  
53 metabolic rates by altering biochemical reactions' kinetics and energies. According to  
54 basic metabolic theory, all organisms possess a survival thermal window, where  
55 increasing temperature increases reaction rates until an optimal level is reached  
56 (Angilletta, 2009). Beyond this threshold, further increases in temperature cause  
57 physiological stress (e.g. protein denaturation), resulting in a steep decline in metabolic  
58 rates and consequently in biological processes (see Fig. 1 in Kingsolver, 2009) such as  
59 growth, development and feeding activity (Angilletta, 2009; Kingsolver, 2009; Mertens  
60 et al., 2015). In fact, due to differential effects on autotrophic and heterotrophic  
61 metabolisms, metabolic theory of ecology (MTE) dictates that producers will show a  
62 weaker response to temperature than consumers (Yvon-Durocher et al., 2010). Thus, as  
63 individual changes lead to community effects (Mertens et al., 2015), small increases in  
64 temperature are predicted to strengthen top-down control by herbivores on marine  
65 primary producers, as a consequence of higher herbivore consumption rates relative to  
66 macroalgal production (Eklöf et al., 2012; O'Connor, 2009). Furthermore, it is also  
67 possible that marine communities find an equilibrium by balancing herbivore grazing  
68 and algal biomass growth, through compensatory mechanisms which allow for  
69 community stabilization (Connell and Ghedini, 2015).

70 The dissolution of atmospheric CO<sub>2</sub> into the oceans alters seawater carbonate system  
71 and the associated drop in pH levels is known as ocean acidification. Since the past

72 century, atmospheric CO<sub>2</sub> concentrations increased to approximately 400 µatm and are  
73 predicted to reach 1000 µatm by the end of this century, with a corresponding drop of  
74 0.14-0.35 units in mean ocean pH (IPCC, 2013). Calcifying animals and algae are  
75 deemed as the most imperiled marine organisms due to deregulation of acid-base  
76 processes, decrease in calcification rates and/or increase in calcium carbonate  
77 dissolution, and hypercapnic growth inhibition (Byrne, 2011; Koch et al., 2013; Kroeker  
78 et al., 2011). Non-calcifying organisms register quite different responses across taxa  
79 (Connell and Russell, 2010; Kroeker et al., 2010; Poore et al., 2013), since some species  
80 of crustaceans and fish show aptitude to partially offset the negative effects of  
81 acidification through mechanisms of acid-base compensation, changes in metabolism,  
82 energy reallocation and/or increase in mobility (Larsen et al., 1997; Melzner et al.,  
83 2009; Widdicombe and Spicer, 2008; Wood et al., 2008). For seaweeds, dissolved CO<sub>2</sub>  
84 is normally used as a substrate for photosynthesis, and non-calcareous species may  
85 benefit from increases of this resource (Koch et al., 2013). Nevertheless, some species,  
86 e.g. *Ulva rigida*, are unaffected by increased CO<sub>2</sub>, as present day carbon concentrations  
87 already saturate its photosynthetic and growth physiological processes (Rautenberger et  
88 al., 2015). Identically to warming effects, ocean acidification may affect species  
89 interaction strength due to differential sensitivities to pH changes, potentially cascading  
90 into alterations in species competition dynamics and trophic interactions (Falkenberg et  
91 al., 2013; Hepburn et al., 2011; Kroeker et al., 2012).

92 To date, most research performed examining climate change impacts of marine  
93 systems have used multi-level single stressor (Connell et al., 2013; Falkenberg et al.,  
94 2013; O'Connor, 2009), straightforward two-stressors/two-levels (2 x 2) (Connell and  
95 Russell, 2010; Gaitán-Espitia et al., 2014; Veteli et al., 2002), or single species  
96 experimental designs (see Wernberg et al., 2012 for a review). However, climate change

97 will create new complex physicochemical scenarios characterized by multi-levels of  
98 stressors interacting. It is critical to improve our ecological knowledge on the potential  
99 combined effects of global stressors not only on different trophic levels, but also on  
100 interacting communities to strengthen our capacity in predicting future impacts of  
101 climate change on marine ecosystems (Poore et al., 2013). Whilst an increase in top-  
102 down control is likely under warming alone (López-Urrutia et al., 2006; O'Connor,  
103 2009), forecasting impacts on macroalgae-herbivore interactions elicited by temperature  
104 and acidification effects is far more complex, given the non-linear variation in organism  
105 metabolic rates across stressor gradients (Mertens et al., 2015). Further increasing  
106 temperature (Mertens et al., 2015) and/or exposure to multiple stressors (Ghedini et al.,  
107 2015a) are reported to overcome the compensatory/grazing metabolic threshold, leading  
108 to shifts in community regimes, from top-down (herbivore-regulated) to bottom-up  
109 (algae- or nutrient-regulated) systems. Moreover, studies comparing mono-specific  
110 herbivore populations *versus* mixed herbivore populations under climate change  
111 scenarios are scarce (Alsterberg et al., 2013), but essential to understand community  
112 effects on natural systems. Within this context, here we studied how distinct CO<sub>2</sub>  
113 concentrations across a temperature gradient would differently impact the survival and  
114 consumption rates of two common herbivore species from the rocky intertidal; the non-  
115 calcifying amphipod species *Melita palmata* (Montagu, 1804), and the calcifying  
116 gastropod species *Gibbula umbilicalis* (da Costa, 1778). Additionally, we tested how  
117 these climate stressors would alter algal biomass of an abundant rocky intertidal algal  
118 species, *Ulva rigida* (Agardh, 1823). Finally, we hypothesized that community  
119 dynamics, i.e. macroalgae-herbivore interaction strength, would be interactively  
120 modified by temperature and CO<sub>2</sub>. Simultaneously, we investigated if grazer species

121 identity may also play a role in shaping community response, due to differential effects  
122 on grazers' survival and consumption rates.

123

## 124 **Methods**

### 125 *Study species*

126 Gastropods are considered efficient grazers (Jernakoff and Nielsen, 1997), generally  
127 grazing larger algal portions than amphipods (Morrisey, 1988). We used the calcifying  
128 gastropod *Gibbula umbilicalis* as a large grazer species and the non-calcifying  
129 amphipod *Melita palmata* as a small-bodied grazer (~1/10 biomass of *G. umbilicalis*) to  
130 assess the joint effects of different climate change stressors in macroalgae-herbivore  
131 interactions. Both macroinvertebrate species are common grazers from the western  
132 Atlantic intertidal rocky coast of the Iberian Peninsula. They can be found associated to  
133 mixed macroalgal beds, including large accumulations of *Ulva spp.*, searching for food  
134 and refuge during low intertidal. The green seaweeds from the *Ulva* genus are very  
135 common opportunistic primary producer in shallow coastal waters and tidal pools in  
136 rocky shore communities (Aníbal et al., 2007).

137 The three species (i.e. *G. umbilicalis*, *M. palmata* and *U. rigida*) were collected on  
138 the same date at Viana do Castelo (North of Portugal, 41°41'44"N, 8°51'2"W), and were  
139 later separated by hand in the laboratory. To select our experimental gradient of  
140 temperatures, we registered water temperature from the sampling location through two  
141 continuous temperature data loggers (Tidbit V2 Onset HOB0®) installed at two mid-  
142 shore rockpools, during the month of August, 2013 (date of this study). Daily average  
143 temperature in the seawater of these rockpools ranged from  $14.96 \pm 0.72$  °C to  $17.80 \pm$   
144  $2.11$  °C (mean  $\pm$  SD) depending on night/day phase, with 13.28 °C and 25.23 °C  
145 recorded as the absolute minimum and maximum values, respectively. This large



146 gradient of temperatures in rock pools from this region results from a combination of  
147 frequent summer upwelling events (Lemos and Pires, 2004), and prolonged exposure to  
148 the sun without water exchange during low tides. As we were interested in investigating  
149 realistic interactions between pH and temperature, CO<sub>2</sub> concentrations followed the  
150 “business-as-usual” predicted scenario for 2100 (IPCC, 2013) and mean experimental  
151 temperatures were chosen within the thermal limits of *G. umbilicalis* (Evans 1948), *U.*  
152 *rigida* (Steffensen 1976) and *M. palmata* (Obenat et al. 2006). We used adult  
153 individuals of amphipods ( $0.96 \pm 0.19$  cm, mean length measured from the tip of the  
154 head to the telson) and gastropods ( $1.05 \pm 0.18$  cm, mean length measured from the tip  
155 of the operculum to the furthest point in the horizontal plane).

156

#### 157 *Experimental mesocosm setting*

158 Experimental mesocosm (closed system) consisted of eight transparent PVC tanks  
159 (approx. 100 L, 50 x 50 x 40 cm, hereafter referred as treatment tanks) filled with  
160 seawater and subjected to eight crossed treatment combinations of two CO<sub>2</sub> levels: ~380  
161  $\pm 30$   $\mu$ atm (ambient) and ~830  $\pm 45$   $\mu$ atm (increased), and four temperature levels  
162 (mean  $\pm$  SD, gradient):  $13.5 \pm 2.0$  °C (low),  $16.1 \pm 2.0$  °C (midlow),  $19.9 \pm 3.0$  °C  
163 (midhigh), and  $22.1 \pm 2.0$  °C (high) (see Figure S1). As mean rock pool temperature  
164 was approximately 16 °C (see also Cacabelos et al. 2013 for confirmation of similar  
165 values), midlow treatment was acknowledged as mean natural temperature conditions.  
166 Temperature was controlled through titanium aquarium heaters, maintained and  
167 adjusted by AT Control devices (Aqua Medic®). Seawater pH was maintained by  
168 manipulating seawater carbonate chemistry, constantly monitored and registered with  
169 Aqua Medic pH electrodes. Each treatment tank (n = 8) connected to a separated PVC  
170 header tank (100 L of capacity, n = 8), where the incorporation of enriched CO<sub>2</sub> /

171 normal air was performed. To adjust pH, a pH-stat system (Aqua Medic<sup>®</sup>, AT Control)  
172 injected certified CO<sub>2</sub>-enriched air (Air Liquide) through solenoid valves to down  
173 regulate pH, and normal air to up regulate pH. Seawater carbonate chemistry was  
174 calculated based on, temperature, salinity, total alkalinity (assessed  
175 spectrophotometrically at 595 nm) and pH<sub>t</sub> measurements (Sarazin et al., 1999) (Table  
176 S1). As such, pH<sub>t</sub> was quantified through a Metrohm pH meter (826 pH mobile,  
177 Metrohm, Filderstadt, Germany) connected to a glass electrode (Schott IoLine, SI  
178 analytics, ± 0.001), which was calibrated with 2-aminopyridine-HCl (AMP) and TRIS-  
179 HCl (TRIS) seawater buffers following Dickson et al. (2007). Total carbonate and pCO<sub>2</sub>  
180 (Table S1) were calculated using CO2SYS software (Lewis and Wallace, 1998), with  
181 equilibrium constants (Mehrbach et al., 1973) refitted by Dickson and Millero (1987).  
182 Abiotic conditions between header and treatment tanks were secured using submersible  
183 pumps (570 l h<sup>-1</sup>), with water permanently exchanged to maintain pH treatments, using  
184 one submersible pump (3000 l h<sup>-1</sup>) and gravitational force. Within each treatment tank,  
185 water exchange was enhanced through water tubing (smaller submersible pump 570 l h<sup>-1</sup>)  
186 that assured all abiotic variable conditions were similar between cylinders.

187 Each treatment tank contained mechanical and biological filtering, as well as twelve  
188 transparent cylinders (D 0.33 x H 0.10 m, ~2 l, 0.085 m<sup>2</sup> cylinder base area), i.e.  
189 experimental units, consisting of four different grazer levels (G: *Gibbula umbilicalis*,  
190 M: *Melita palmata*, G+M: both species together, and C: controls with no grazers, n = 3)  
191 interspersed through the treatment tank (Figure S1). The top of each cylinder was  
192 covered with a 1.0 mm mesh to avoid animals from escaping, while still enabling water  
193 circulation and light entry. Nutrients (1 ml of 42.50 g l<sup>-1</sup> NaNO<sub>3</sub> solution and 1 ml of  
194 10.75 g l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub> solution per liter of seawater) were added to each tank every two  
195 days, and water was renewed manually when salinity increased to 36 (35.5 ± 1.0 PSU;

196 mean  $\pm$  SD, n = 8). Temperature, pH and salinity were monitored three times a day with  
197 the use of a data-logger. The experimental setup was placed inside a shadow greenhouse  
198 under natural light and photoperiod, with consequently reduced incident light inside,  
199 mimicking commonly found subsaturating light conditions on rocky pools (Guidone et  
200 al., 2012; O'Connor, 2009).

201

202 *Experiment I: Warming and acidification effects on grazers consumption rates*

203 We performed a two-day (i.e. 48h) experiment to assess grazer consumption rates for  
204 mono-specific and mixed grazer combinations (O'Connor, 2009). This experiment was  
205 preceded by a 24h algae-free period for grazer acclimatization (algae were acclimated in  
206 separate cylinders) to avoid the influence of past diet and ensure algae consumption  
207 during the experiment (Swanson & Fox, 2007). We used 3-replicated cylinders with  
208 four levels of grazers (density in cylinder = 0.009 ind / m<sup>2</sup>): *G. umbilicalis* (hereafter G,  
209 n = 10), *M. palmata* (hereafter M, n = 10), both species together (hereafter G+M, n = 5  
210 + 5), and control (hereafter C, no grazers). At the end of the 24 hour acclimatization  
211 period, algae were spun 25 revolutions in a salad spinner to remove excess water and  
212 ~3.015  $\pm$  0.041 g (mean  $\pm$  SD, density in cylinder = 0.028 g algae / m<sup>2</sup>) of algal blotted  
213 wet weight (bww) were inserted in each cylinder. While in the cylinders, algae were  
214 strapped to weights and left swinging inside the cylinder, mimicking natural rock pool  
215 conditions. After the 48 hour grazing trial, portions were removed, blotted and weighted  
216 again as above. Dead animals were recorded at the end of the experiment.

217

218 *Experiment II: Warming and acidification effects on interaction strength*

219 We performed a longer (seven-day or 168 hours) experiment with new animals and  
220 algal portions to assess differences in per capita macroalgae-herbivore interaction

221 strength, i.e. individual grazer pressure per algal biomass growth ratio. A 24h period of  
222 acclimatization with algae was performed (Eklöf et al., 2015), and at the beginning of  
223 the experiment, algae were added, mimicking natural conditions where seaweed is  
224 abundant (Cacabelos et al., 2013; O'Connor, 2009). Three-replicated cylinders were  
225 used, with 6 grazers for each level of grazer treatment (density in cylinder = 0.014 ind /  
226 m<sup>2</sup>): G (n = 6), M (n = 6), G+M (n = 3 + 3) and control (C, no grazers). Grazer numbers  
227 were reduced to avoid excessive competition for habitat and food. At the beginning of  
228 the experiment,  $\sim 7.030 \pm 0.025$  g (mean  $\pm$  SD, density in cylinder = 0.012 g algae / m<sup>2</sup>)  
229 of blotted wet weight portions of algae were strapped to weights and inserted in each  
230 cylinder. After seven days, algae were retrieved, blotted and weighted. The number of  
231 dead animals was recorded at the end of the experiment.

232

### 233 *Data Analysis*

234 Survival rates in both experiments were assessed according to the number of living  
235 *G. umbilicalis* and *M. palmata* individuals (and the sum of both in the case of G+M  
236 treatment) present at the end of each experiment, and transformed into survival rate (%)  
237 per cylinder. Changes in algal biomass were estimated through the difference of initial  
238 and final values of algal wet biomass in experiments. Consumption rates in Experiment  
239 I was calculated using the following formula based on Taylor and Brown (2006):

240

$$241 \text{ Consumption} = ( T_i * ( C_f / C_i ) - T_f ) / ( n_{\text{bio}} * t ),$$

242

243 Where  $T_i$  is the initial algal bww,  $T_f$  is the final algal bww,  $C_i$  is the initial control  
244 algal bww,  $C_f$  is the final control algal bww,  $n_{\text{bio}}$  is the added biomass of living grazers  
245 in each cylinder at the end of the experiment and  $t$  is the time elapsed in the experiment

246 (2 days). Thus, consumption rates are expressed in grams of algae consumed per  
247 invertebrate biomass per day. Biomass was extrapolated from a referenced length-  
248 weight regression based on *M. palmata* individuals' total length (Grilo et al., 2009;  
249 Pardal et al., 2002). Likewise, *G. umbilicalis* biomass was extrapolated by measuring  
250 the longest vertical axis and fitted in a referenced length-weight regression (Robinson et  
251 al., 2010).

252 In Experiment II, in addition to survival rates and changes in algal biomass,  
253 alterations in community dynamics, i.e. grazing pressure and simultaneous macroalgal  
254 growth, were also analyzed. To assess the strength of macroalgae-herbivore interaction  
255 we used the Dynamic Index (Berlow et al., 1999; Mertens et al., 2015; O'Connor, 2009;  
256 Wootton and Emmerson, 2005) modified from Wootton (1997), with the following  
257 formula:

258

$$259 \quad DI = \ln ( N / D ) / ( n * t ),$$

260

261 where DI is the Dynamic Index, N is algal wet biomass with grazers, D is the algal  
262 wet biomass on treatment without grazers, n is the number of living grazers in the  
263 cylinder, and t is the known period of time elapsed (i.e. seven days). This index  
264 measures interaction strength, i.e. the absolute value of daily per capita interaction  
265 strength, accounting for differences in algal growth rates with and without herbivores  
266 (O'Connor 2009). Negative interaction strength values implicate that one species  
267 reduces the abundance of the other species; therefore, lower values indicate stronger  
268 interactions (i.e. high grazer pressure). This index translates survival rates and metabolic  
269 alterations provoked by climate change stressors into an ecological response (O'Connor  
270 2009). Compared to other methods, DI does not assume equilibrium between algae and

271 grazers, which is convenient for relatively short experiments as ours (Berlow et al.,  
272 1999).

273 Changes in survival rates, algal biomass, consumption rates and DI were analyzed  
274 separately through a distance-based permutational multivariate analysis of variance  
275 (PERMANOVA, Anderson 2001). We calculated distance resemblance matrices using  
276 Euclidean dissimilarity measures based on untransformed data. PERMANOVA was run  
277 with 4999 permutations to obtain  $p$  values under unrestricted permutation of raw data  
278 (Anderson 2001), using CO<sub>2</sub> (2 levels; ambient and increased), temperature (4 levels:  
279 low, midlow, midhigh and high) and grazers (4 levels: G, M, G+M and C) as fixed  
280 orthogonal factors ( $n = 3$ ). The permutational approach was used because the data were  
281 non-normally distributed even after transformations (Wernberg et al., 2008). Only  
282 significant effects ( $p < 0.05$ ) were further investigated through a series of pair-wise  
283 comparisons using the appropriate terms in the model (Anderson et al. 2008). All the  
284 multivariate analyses were performed with PRIMER 6 & PERMANOVA+ package.

## 285 **Results**

286

### 287 *Experiment I: Warming and acidification on grazer consumption rates*

288

289 After 48h, survival rates differed significantly depending on temperature and grazer  
290 treatments (Table 1). Specifically, survival rates decreased from lower (13.5 °C and 16.1  
291 °C) to higher (19.9 °C and 22.1 °C) temperatures ( $p < 0.001$ , Figure 1, see Table S2 for  
292 pair-wise comparisons). Concerning grazer treatments, survival rates were  
293 comparatively higher in G (90%), and lower in M (~40%), while G+M registered  
294 intermediate survival rates (~70 %) (Figure 1, Table S2).

295 Algal biomass was significantly altered by temperature and the interaction between  
296 CO<sub>2</sub> and grazers (Table 1, Figure 2). Thus, at high temperature (22.1 °C) algal biomass  
297 loss was reduced compared to biomass loss reported for the two lower temperature  
298 levels, 13.5 °C and 16.1 °C (Figure 2a, Table S3). G registered higher biomass loss than  
299 M, and increased CO<sub>2</sub> amplified these effects (Figure 2b, Table S3). In grazer control  
300 treatments, i.e. C, algal biomass increased: 0.1 g (from initial 3 g) per day  $\approx$  3 % per  
301 day.

302 Consumption rates were significantly affected by the triple interaction between CO<sub>2</sub>,  
303 temperature and grazer (Table 1). Multiple pairwise comparisons revealed that  
304 consumption rates in M were always higher than in G and G+M, regardless of  
305 temperature or CO<sub>2</sub> (Figure 3, Table S4). Comparing CO<sub>2</sub> treatments, at 13.5 °C, G+M  
306 displayed lower consumption rates in increased CO<sub>2</sub> (Figure 3, Table S4). Under  
307 ambient CO<sub>2</sub> (Figure 3a), G and G+M consumption rates dropped across the  
308 temperature gradient, whereas M consumption rates increased (Figure 3, Table S4).  
309 Lastly, under increased CO<sub>2</sub>, temperature did not different consumption rates in any  
310 grazer treatment (Figure 3b, Table S4).

311

### 312 *Experiment II: Warming and acidification effects on interaction strength*

313

314 After 7 days (i.e. 168h), survival rates were significantly affected by variations in  
315 CO<sub>2</sub> and the interaction between temperature and grazers (Table 2). Lower survival  
316 rates were reported under increased CO<sub>2</sub> (SNK test,  $p < 0.001$ , Figure 4a). Moreover,  
317 survival rates also dropped with increasing temperature in all grazer treatments (Figure  
318 4b, Table S5). Specifically, all grazer treatments showed higher survival rates at 13.5 °C

319 than at 19.9 °C and 22.1 °C. Comparing grazer treatments, G generally showed  
320 significantly higher survival rates (Figure 4b, Table S5).

321 Algal biomass in Experiment II was significantly affected by a triple interaction of  
322 CO<sub>2</sub>, temperature and grazer (Table 2, Figure 5). Temperature and CO<sub>2</sub> showed no  
323 effect on grazer control treatments, which consistently revealed a ~20 % increase (from  
324 initial 7 g, 0.2g increase per day  $\simeq$  3 % per day; 3% per day \* 7 days  $\simeq$  20 %) in algal  
325 biomass at the end of 7 days (Table S6). Algal biomass loss peaked at 19.9 °C under  
326 ambient CO<sub>2</sub>, while increased CO<sub>2</sub> led to higher algal biomass loss at 16.1 °C (Table  
327 S6). Moreover, extreme temperatures (13.5 °C and 22.1 °C) consistently registered algal  
328 biomass growth, in contrast to algal loss verified in intermediate temperatures (16.1 °C  
329 and 19.9 °C). Under ambient CO<sub>2</sub> and 13.5 °C, *Ulva rigida* growth was higher in M  
330 compared to G+M (SNK test,  $p < 0.05$ , Table S6).

331 The Dynamic Index (DI) was interactively affected by CO<sub>2</sub> and temperature (Table  
332 2, Figure 6). Thus, under increased CO<sub>2</sub> a temperature increase from 13.5 °C to 16.1 °C  
333 led to an overall DI minimum, i.e. largest top-down control (SNK test,  $p < 0.01$ , Table  
334 S7), while at ambient CO<sub>2</sub> no significant effects in DI were detected (Figure 6). Under  
335 increased CO<sub>2</sub>, further temperature increase (16.1 °C to 19.9 °C and 22.1 °C) led to a  
336 consecutive increase in DI values, i.e. a reduction of top-down interaction strength  
337 (Figure 6, Table S7). Conversely, under ambient CO<sub>2</sub>, the strongest top-down  
338 interaction (i.e. low DI, high grazer pressure) occurred at 19.9 °C. Once again this  
339 significant trend was reverted at 22.1 °C, and DI values indicating bottom-up control  
340 were registered (SNK test,  $p < 0.001$ ; Figure 6, Table S7). No grazer treatment effects  
341 were observed in the DI index (Table 2).

## 342 Discussion



343 Our results showed that grazers' survival and consumption rates were differently  
344 affected by warming and acidification. Additionally, temperature showed immediate  
345 effects (Experiment I, two days) on survival rates, whereas acidification-related effects  
346 emerged later on (Experiment II, seven days). When co-occurring, stressor impacts were  
347 additive and independent, hence no interactive climate stressor effects were found on  
348 survival rates. Increased CO<sub>2</sub>, i.e. hypercapnia-linked, mortality is known to occur as a  
349 consequence of prolonged animal inactivity and/or starvation due to metabolic  
350 suppression, which explains why CO<sub>2</sub> effects were only detected after seven days  
351 (Kurihara et al., 2008; Langenbuch and Pörtner, 2004; Spicer et al., 2007). Thus, these  
352 results strengthen the claim that warming is the strongest stressor, outweighing  
353 acidification effects on animal survival (see Wernberg et al. 2012 for a review). the  
354 relatively high mortality verified was likely caused by lower animal physiological  
355 tolerances to fixed stressor (e.g. temperature) levels in comparison to variable stressor  
356 levels (Benedetti-Cecchi et al., 2006; Vasseur et al., 2014). Contrary to natural  
357 fluctuating conditions (i.e. where stressor levels vary over time), our experimental  
358 conditions comprised of fixed stressor treatments (i.e. constant thermal and pH  
359 conditions with low intra-treatment variance), which likely contributed to increased  
360 physiological stress and mortality (Stillman, 2002).

361 Herbivore performance analysis also revealed striking differences between both  
362 grazers tested. Molluscs are usually considered more sensitive to warming, and  
363 especially acidification, than crustaceans (Harvey et al., 2013). In theory, due to  
364 naturally higher basal metabolism (see Peck et al., 2009), *M. palmata* may be closer to  
365 its metabolic peak (i.e. "optimal" level) than *G. umbilicalis*, thus overcoming the  
366 optimal metabolic threshold with shorter increases in stressors. Consumption rates as  
367 impacts on algal biomass were similar to those reported by Giannotti and McGlathery

368 (2001) for gastropod (around 0.01-0.02 grams of algae per individual and day) and by  
369 Cruz-Rivera and Hay (2001) for amphipods (around 0.004-0.01 grams of algae per  
370 individual and day) grazing on *Ulva* sp. Thus, *G. umbilicalis* showed higher impact on  
371 algae biomass, with natural temperature (16.1 °C) and increased CO<sub>2</sub> amplifying algal  
372 biomass loss. Nevertheless, we found that *M. palmata* is actually a stronger grazer per  
373 biomass than *G. umbilicalis*, likely due higher metabolic rates. *G. umbilicalis* inherently  
374 higher biomass also mathematically justifies why consumption rates per biomass in  
375 mixed species treatments closely matched those reported for *G. umbilicalis*. Thus,  
376 according to our results, increases in temperature (but also in CO<sub>2</sub>) will benefit *G.*  
377 *umbilicalis* by lowering *M. palmata* survival, while increasing grazing pressure on algal  
378 biomass, as predicted by the metabolic theory of ecology (MTE; Kingsolver, 2009).

379 Concerning abiotic effects on seaweed biomass, our results are in line with previous  
380 research, reporting no acidification effects on *U. rigida* growth (Rautenberger et al.,  
381 2015), but differ from the previously reported temperature dependence for the genus  
382 *Ulva* (Steffensen, 1976). In our study, controls (only algae, no grazers) showed similar  
383 algal biomass increase under warming and acidification conditions, in both experiments  
384 (~3 % growth per day). As such, mean algal growth rate reported here is lower than  
385 expected under perfect light intensity laboratorial conditions (20 % growth per day in  
386 Rautenberger et al., 2015). However, it is similar to results verified in field experiments,  
387 as well as for laboratorial settings mimicking subsaturating light conditions (e.g. ~5 %  
388 growth per day in Guidone et al., 2012; see also O'Connor, 2009). We suggest that the  
389 lack of algal response to climate stressors and the algal growth rates registered here  
390 were caused by comparatively reduced solar irradiation (Rautenberger et al., 2015). This  
391 reduction was a direct consequence of the greenhouse set-up housing, further amplified  
392 perhaps by the use of a 1 mm mesh on the experimental cylinders (to prevent grazers

393 from escaping). Moreover, warming-related algal growth may have been potentially  
394 concealed by undetected temperature-induced algal decomposition (Schiel et al., 2004).

395 After seven days (Experiment II), algal biomass changes in treatments including  
396 grazers showed the expected profile across the temperature gradient (Kingsolver, 2009;  
397 Mertens et al., 2015). However, maximum algal biomass loss also depended on CO<sub>2</sub>  
398 conditions. Although interaction strength may increase with higher temperature and  
399 CO<sub>2</sub>, as shown by previous studies (Alsterberg et al., 2013; O'Connor, 2009), the  
400 response patterns are indeed more complex than a direct linear antagonistic or  
401 synergistic effect (Ghedini et al., 2015a). These non-linear CO<sub>2</sub>-dependent responses to  
402 temperature gradients were clearly displayed in the dynamic index profiles. As it is,  
403 grazing pressure increased over increasing temperature, until survival rates decreased to  
404 the point of soothing grazer pressure on macroalgae (i.e. overcoming the community  
405 stability threshold), which significantly weakened macroalgae-herbivore interactions,  
406 and ultimately shifted community dominance. As expected, herbivores were shown to  
407 be more susceptible to changes in temperature than primary producers (López-Urrutia et  
408 al., 2006). Moreover, increased CO<sub>2</sub> stimulated community response by accelerating the  
409 interaction strength profile across warming scenarios. Thus, as predicted by Ghedini et  
410 al. (2015a), the occurrence of simultaneous stressors lead to a bottom-up controlled  
411 community earlier in the temperature gradient.

412 The observed warming and acidification effects were consistent in the three different  
413 combinations where grazers were used, i.e. previously detected grazer identity effects  
414 for other parameters were concealed in the dynamic index analyses. Thus, our results  
415 did not support the claim that grazing activity by different grazer species would modify  
416 the strength of top-down control on seaweeds (O'Connor 2009). Most likely our  
417 election of the herbivores, without a clearly defined stronger grazer (i.e. *M. palmata* was

418 the stronger grazer per biomass, but produced a comparatively smaller impact on algal  
419 biomass, and was also the most sensitive to the climate stressors) was the main cause  
420 for the verified grazer homogeneity. Moreover, our experimental set-ups ran through  
421 relatively short time spans (up to seven days), so extrapolating or generalizing  
422 conclusions to natural systems may be inaccurate. However, it is important to note that  
423 studies that were prolonged for longer periods of time (two to five weeks), generally led  
424 to stronger interactions between climate change stressors (e.g. Alsterberg et al., 2013;  
425 Christensen et al., 2011; Vasseur et al., 2014). Furthermore, the strength of macroalgal-  
426 herbivore interactions is reported to increase from 11 to 17 days (Ghedini et al., 2015b;  
427 Mertens et al., 2015; O'Connor, 2009). Therefore, we suggest that grazer identity  
428 effects may still arise in the future ocean as a consequence of starkly different survival  
429 and consumption rates.

430 Future climate change is expected to modify the fundamental top-down control  
431 exerted by herbivore species feeding on macroalgae (Eklöf et al. 2012, this study).  
432 Warming and acidification can interact and play a major role in differentially driving  
433 rocky intertidal communities' structure and functioning. Extreme increases in both  
434 stressors, or higher frequencies of extreme weather events (Kroeker et al., 2011), can  
435 largely affect the survival rates of typical grazers. Taking advantage of lower thermal  
436 sensitivity, opportunistic algal species like *U. rigida* might grow freely, disrupt  
437 ecosystem equilibrium, and promote ecological shifting from top-down to bottom-up  
438 regulated communities (Connell and Russell, 2010; Veteli et al., 2002). Conversely,  
439 moderate increases in acidification or warming might lead to higher grazing pressure as  
440 long as these stressors do not exceed metabolic grazers' thermal and acid-base limits  
441 (Alsterberg et al., 2013; O'Connor, 2009), and lower algal biomass as a consequence of  
442 stronger top-down control (i.e. increased interaction strength). Thus, understanding

443 whether macroalgal and herbivore responses to different climate change stressors are  
444 synchronous is fundamental to assess the future strength of top-down control in marine  
445 systems. We also suggest that the characteristics and composition of intertidal grazer  
446 assemblages may play a relevant role in macroalgae-herbivore interactions, inducing  
447 differences in grazing pressure on macroalgae, mainly due to species-specific  
448 differences in survival and algal consumption rates. Longer exposures in mesocosm and  
449 field experiments focusing on the combined effects of different climate stressor  
450 gradients, and using diverse assemblages of herbivores and macroalgal species, are  
451 needed to underpin forecasts on how macroalgae-herbivore interactions will be affected  
452 by global change in the future.

453

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685 **Figures & Tables**

686

687 **Figure 1.** Survival rates of *G. umbilicalis* and *M. palmata* individuals present (% , mean  
688 +SE) at the end of Experiment I. Data is displayed according to significant factors (non-  
689 significant factor are averaged, see Table 1): a) temperature (low: 13.5 °C, midlow: 16.5  
690 °C, midhigh: 19.9 °C, high: 22.1 °C), b) grazer treatments (G: *G. umbilicalis*, M: *M.*  
691 *palmata*, G + M: *G. umbilicalis* + *M. palmata*. Different letters represent significant  
692 differences.

693

694 **Figure 2.** Changes in mean (+ SE) algal biomass (*U. rigida* g, blot wet weight per day)  
695 in the end of Experiment I. Data is displayed relative to significant factors (non-  
696 interactive factors are averaged, see Table 1): a) temperature (low: 13.5 °C, midlow:  
697 16.5 °C, midhigh: 19.9 °C, high: 22.1 °C), and b) CO<sub>2</sub> (ambient CO<sub>2</sub>: 380 µatm,  
698 increased CO<sub>2</sub>: 830 µatm) and grazer treatments (G: *G. umbilicalis*, M: *M. palmata*, G +  
699 M: *G. umbilicalis* + *M. palmata*, C: No grazers). Different letters represent significant  
700 differences.

701

702 **Figure 3.** Effects on mean (+SE) herbivore consumption rates in the end of Experiment  
703 I. Data is displayed relative to significant factors (non-interactive factors are averaged,  
704 see Table 1): a) grazer (G: *G. umbilicalis*, M: *M. palmata*, G + M: *G. umbilicalis* + *M.*  
705 *palmata*) and CO<sub>2</sub> treatments (ambient CO<sub>2</sub>: 380 µatm, increased CO<sub>2</sub>: 830 µatm), and  
706 b) grazer (G: *G. umbilicalis*, M: *M. palmata*, G + M: *G. umbilicalis* + *M. palmata*) and  
707 temperature treatments (low: 13.5 °C, midlow: 16.5 °C, midhigh: 19.9 °C, high: 22.1  
708 °C). Results expressed as grams of algae (algae biomass) per milligrams of grazers

709 (grazer biomass) contained in the treatment, per day. Different letters represent  
710 significant differences.

711

712 **Figure 4.** Survival rates of *G. umbilicalis* and *M. palmata* individuals present (% , mean  
713 + SE) at the end of Experiment II. Data is displayed relative to significant factors (non-  
714 interactive factors are averaged, see Table 2): a) CO<sub>2</sub> (ambient CO<sub>2</sub>: 380 µatm,  
715 increased CO<sub>2</sub>: 830 µatm), and b) temperature (low: 13.5 °C, midlow: 16.5 °C, midhigh:  
716 19.9 °C, high: 22.1 °C) and grazer treatments (G: *G. umbilicalis*, M: *M. palmata*, G + M:  
717 *G.umbilicalis* + *M. palmata*). Different letters represent significant differences.

718

719 **Figure 5.** Effects of temperature (low: 13.5 °C, midlow: 16.5 °C, midhigh: 19.9 °C,  
720 high: 22.1 °C) and grazer treatments (G: *G. umbilicalis*, M: *M. palmata*, G + M:  
721 *G.umbilicalis* + *M. palmata*, C: No grazers) on mean (± SE) algal growth (*U. lactuca* g,  
722 blot wet weight) at a) ambient CO<sub>2</sub> (380 µatm) and b) increased CO<sub>2</sub> (830 µatm) in the  
723 end of Experiment II. Different letters represent significant differences.

724

725 **Figure 6.** Effects of temperature (low: 13.5 °C, midlow: 16.5 °C, midhigh: 19.9 °C,  
726 high: 22.1 °C) and CO<sub>2</sub> treatments (ambient CO<sub>2</sub>: 380 µatm, increased CO<sub>2</sub>: 830 µatm)  
727 on the Dynamic Index (DI, mean ± SE) as a strength measure of the herbivore-  
728 macroalgae interaction (Experiment II). Data is displayed according to significant  
729 factors (non-significant factor is averaged, see Table 2). Different letters and numbers  
730 represent significant differences.

731

732

733 **Table 1.** Summary of PERMANOVA for the effects of temperature (T, 4 levels: low, midlow, midhigh, high), CO<sub>2</sub> (2 levels: ambient and  
 734 increased), and grazer (G, 3 levels: *G. umbilicalis*, *M. palmata*, *G. umbilicalis* + *M. palmata*) treatments on grazer survival and consumption rates  
 735 for Experiment I. DF: Degrees of freedom, MS: Mean squares. Significant values are in bold ( $p < 0.05$ ).

736

Source of variation	<i>Grazer survival rate</i>				<i>Algal biomass</i>				<i>Consumption rate</i>			
	DF	MS	F	<i>P</i> (perm)	DF	MS	F	<i>P</i> (perm)	DF	MS	F	<i>P</i> (perm)
CO <sub>2</sub>	1	7133.5	14.911	<b>0.0004</b>	1	0.1700	11.852	<b>0.0018</b>	1	0.0002	3.7689	0.0596
T	3	13368	27.944	<b>0.0002</b>	3	0.0410	2.8572	<b>0.0470</b>	3	0.0001	1.2986	0.2862
G	2	3059.4	6.3952	<b>0.0052</b>	3	0.8490	59.190	<b>0.0002</b>	2	0.0102	241.45	<b>0.0002</b>
CO <sub>2</sub> x T	3	538.84	1.1263	0.3466	3	0.0285	1.9904	0.1318	3	0.0001	2.4633	0.0762
CO <sub>2</sub> x G	2	397.38	0.8307	0.4386	3	0.0497	3.4651	<b>0.0256</b>	2	0.0001	1.5419	0.2124
T x G	6	1315.6	2.7500	<b>0.0242</b>	9	0.0226	1.5745	0.1414	6	0.0001	2.0158	0.0814
CO <sub>2</sub> x T x G	6	145.32	0.3038	0.9324	9	0.0110	0.7697	0.6466	6	0.0001	3.2616	<b>0.0106</b>
Residuals	48	478.40			64	0.0143			48	0.0000		
Total	71				95				71			

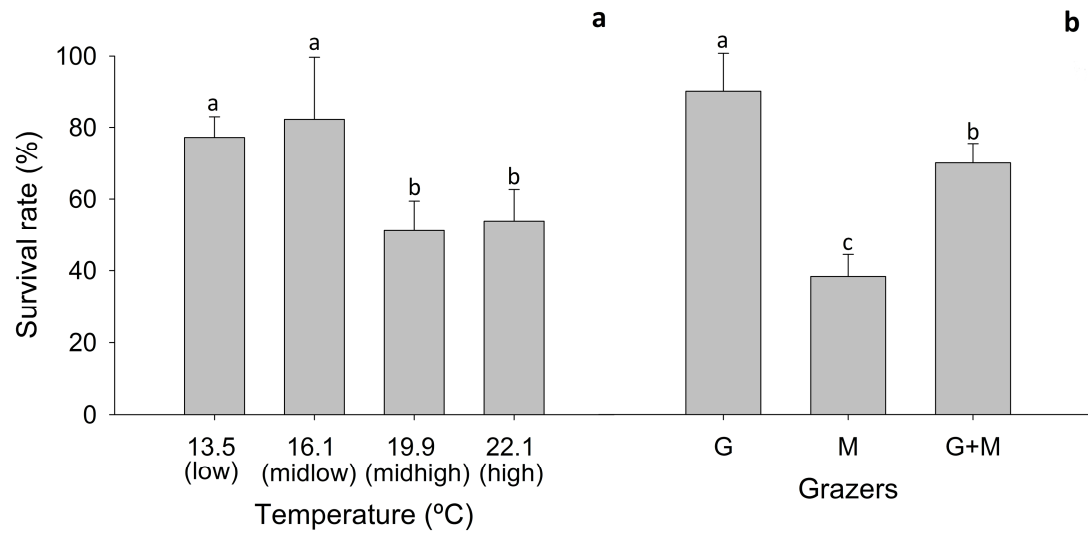
737

738 **Table 2.** Summary of PERMANOVA for the effects of temperature (T, 4 levels: low, midlow, midhigh, high), CO<sub>2</sub> (2 levels: ambient and  
 739 increased), and grazer (3 levels: *G. umbilicalis*, *M. palmata*, *G. umbilicalis* + *M. palmata*) treatments on grazer survival rate, algal growth and  
 740 Dynamic Index for Experiment II. DF: Degrees of freedom, MS: Mean squares. Significant values are in bold ( $p < 0.05$ ).

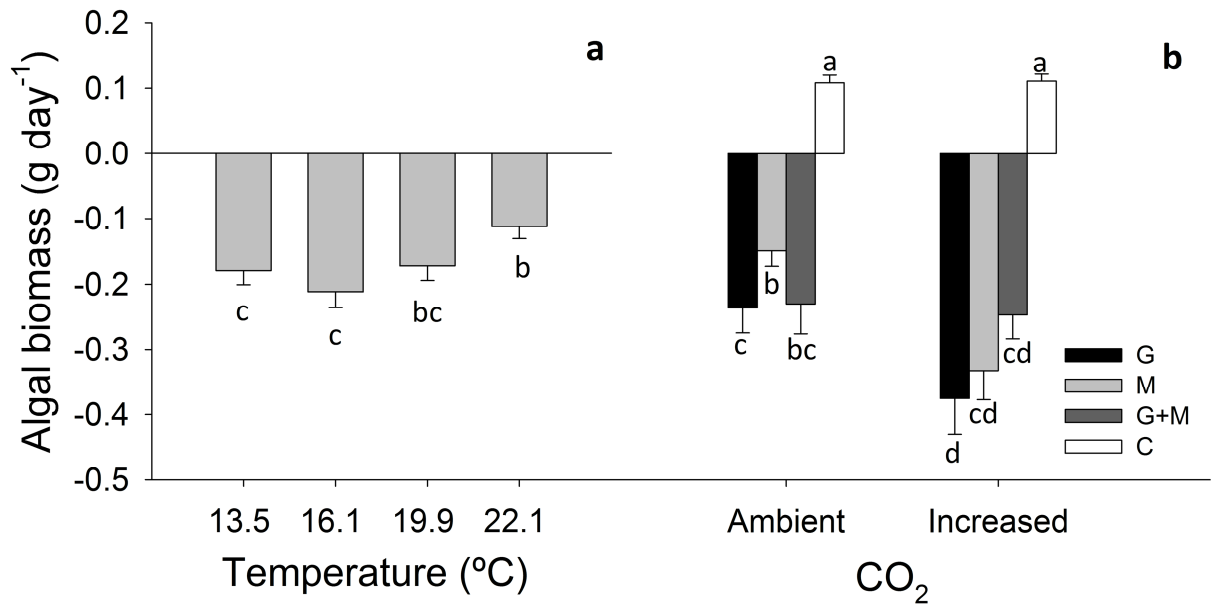
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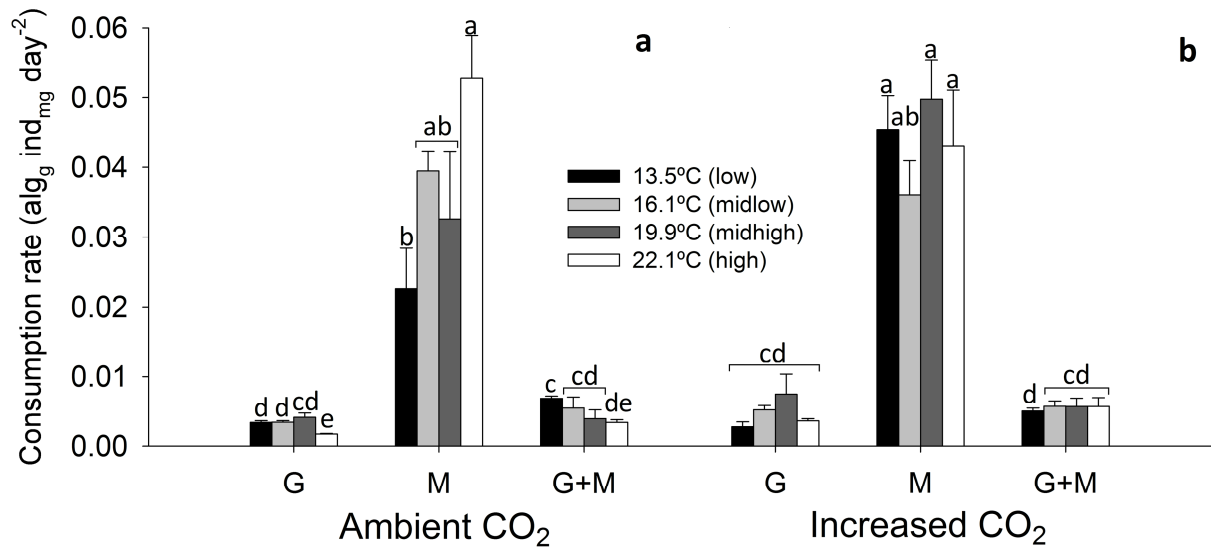
Source of variation	<i>Grazer survival rate</i>				<i>Algal biomass</i>				<i>Dynamic Index</i>			
	DF	MS	F	<i>P</i> (perm)	DF	MS	F	<i>P</i> (perm)	DF	MS	F	<i>P</i> (perm)
CO <sub>2</sub>	1	22.22	0.0465	0.8202	1	0.0010	0.1223	0.7204	1	0.3155	8.0122	<b>0.0042</b>
T	3	4544.4	9.5116	<b>0.0002</b>	3	0.4468	52.9910	<b>0.0002</b>	3	0.8288	21.046	<b>0.0002</b>
G	2	16289	34.093	<b>0.0002</b>	3	0.2097	24.8660	<b>0.0002</b>	2	0.0346	0.8784	0.4376
CO <sub>2</sub> x T	3	277.78	0.5814	0.6246	3	0.2306	27.3470	<b>0.0002</b>	3	0.8202	20.826	<b>0.0002</b>
CO <sub>2</sub> x G	2	72.222	0.1511	0.8544	3	0.0316	3.7510	<b>0.0154</b>	2	0.0499	1.2681	0.2886
T x G	6	833.33	1.7442	0.1294	9	0.0546	6.4809	<b>0.0002</b>	6	0.0325	0.8250	0.5646
CO <sub>2</sub> x T x G	6	394.44	0.8256	0.547	9	0.0447	5.2981	<b>0.0006</b>	6	0.0445	1.1306	0.3576
Residuals	48	477.78			64	0.0084			48	0.0394		
Total	71				95				71			

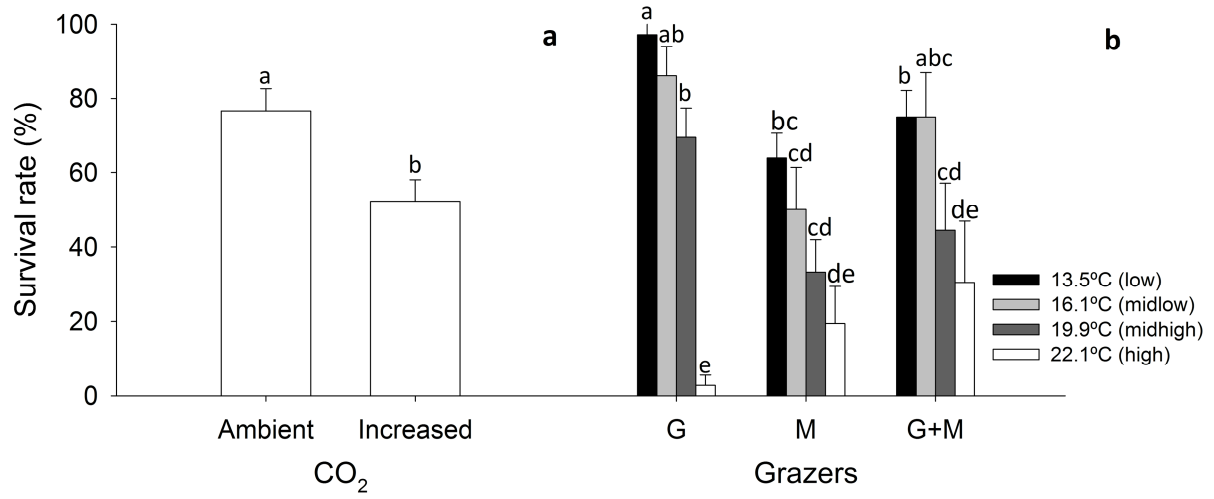
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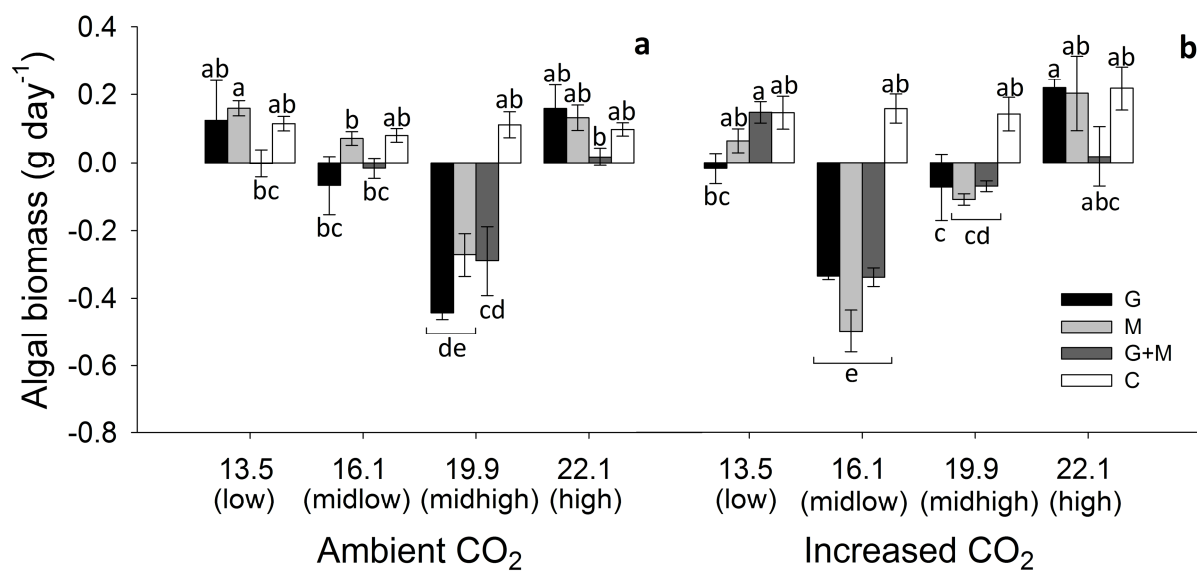


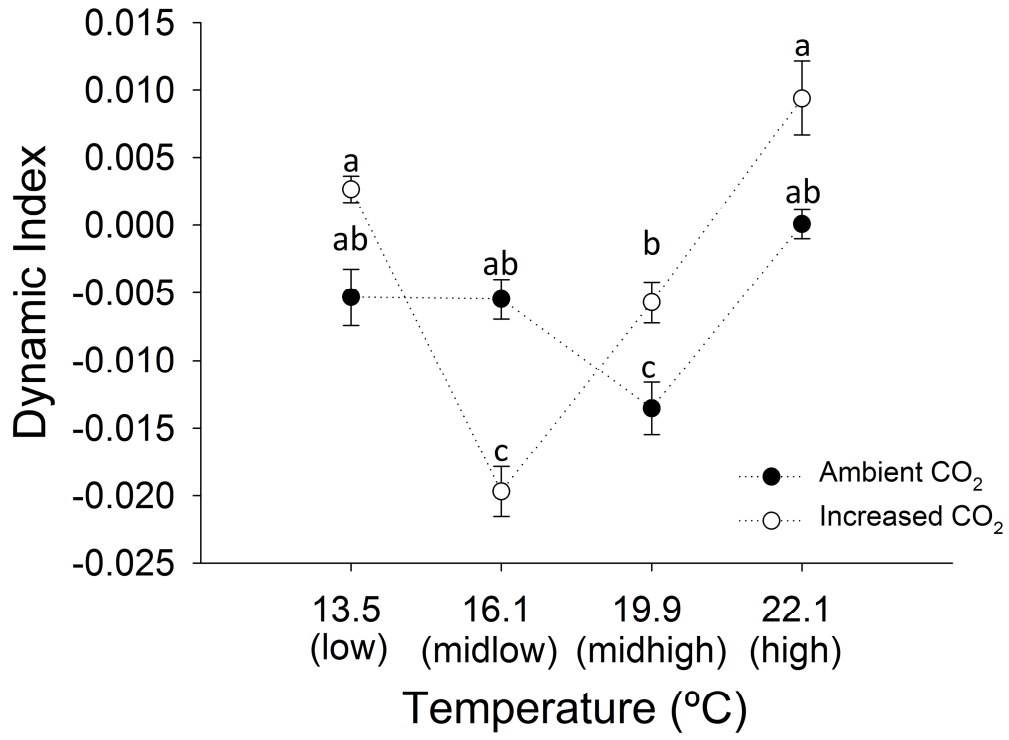












**Highlights**

- Warming and acidification elicited negative effects in grazer's survival rates.
- Macroalgae-herbivore interaction strength was shaped by temperature gradient.
- Acidification shifts thermal optimal metabolic threshold to lower temperatures.
- Grazer identity may modulate macroalgae-herbivore interactions.